

FORM PTO-1390 (REV 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 159-72
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 10/089563 unknown
INTERNATIONAL APPLICATION NO. PCT/JP00/05170	INTERNATIONAL FILING DATE 02/08/2000	PRIORITY DATE CLAIMED 02/08/2000	
TITLE OF INVENTION KITS FOR EXTRACING NUCLEIC ACID AND METHOD OF EXTRACTING NUCLEIC ACID BY USING THE KITS			
APPLICANT(S) FOR DO/EO/US YOSHIHARA, N. et al.			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input type="checkbox"/> The U.S. has been elected by the expiration of 19 months from the priority date (Article 31). 5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 			
<p>Items 11 To 20 below concern document(s) or information included:</p> <ol style="list-style-type: none"> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98. 12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information. PTO Form 1449, 3 sheets of drawings, and 7 pages of Sequence Listing 			

- 2 -

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REC'D PCT/PTO 13 AUG 2002 10/089563

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

YOSHIHARA, N. et al.

Atty. Ref.: 159-72

Serial No. 10/089,563

Group:

US National Phase of PCT/JP00/05170

Filed: April 2, 2002

Examiner:

For: KITS FOR EXTRACTING NUCLEIC ACID AND METHOD OF EXTRACTING
NUCLEIC ACID BY USING THE KITS

* * * * *

August 13, 2002

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

AMENDMENT

Responsive to the Notification dated June 13, 2002 (copy attached), entry and
consideration of the following amendments and remarks are requested.

IN THE SPECIFICATION

Amend the specification as follows:

Insert the attached Sequence Listing in place of the originally-filed copy of the
same.

REMARKS

The specification has been amended to include the attached Sequence Listing.

YOSHIHARA, N. et al.
Serial No. **10/089,563**
US National Phase of PCT/JP00/05170

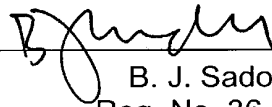
The attached paper and computer readable copies of the Sequence Listing are the same. No new matter has been added. A separate Statement to this effect is attached.

An early and favorable Action on the merits is requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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* * * * *

April 2, 2002

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

In order to place the above-identified application in better condition for
examination, please amend the application as follows:

IN THE SPECIFICATION

Please substitute the following paragraphs in the specification for corresponding
paragraphs previously presented. A copy of the amended specification paragraphs
showing current revisions is attached.

Page 1, before the first line, please insert as a separate paragraph:

This application is the US national phase of international application
PCT/JP00/05170 filed 02 August 2000, which designated the US.

IN THE CLAIMS

Please substitute the following amended claims for corresponding claims previously presented. A copy of the amended claims showing current revisions is attached.

3. (Amended) The nucleic acid isolation kit of Claim 1 wherein the nucleic acid is RNA.
4. (Amended) The nucleic acid isolation kit of Claim 1 wherein the reducing agent is 2-mercaptoethanol or dithiothreitol.
5. (Amended) The nucleic acid isolation kit of Claim 1 wherein the coprecipitant is glycogen or dextran.
6. (Amended) The nucleic acid isolation kit of Claim 1 wherein the protein denaturant is guanidine thiocyanate.
10. (Amended) The method of Claim 7 comprising adding a protein denaturant during alcohol precipitation.

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11. (Amended) The method of Claim 7 wherein the biological component is a body fluid or a blood product.

12. (Amended) The method of Claim 7 wherein the biological sample has a volume of 30 μ l to 100 μ l.

13. (Amended) The method of Claim 7 comprising no step of adding a salt.

14. (Amended) The method of Claim 7 wherein said steps are performed in a single 0.5 ml tube.

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REMARKS

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page(s) is captioned "**Version With Markings To Show Changes Made.**"

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION

Page 1, before the first line, please insert as a separate paragraph:

This application is the US national phase of international application
PCT/JP00/05170 filed 02 August 2000, which designated the US.

IN THE CLAIMS

3. (Amended) The nucleic acid isolation kit of Claim 1-~~or 2~~ wherein the nucleic acid is RNA.

4. (Amended) The nucleic acid isolation kit of ~~any one of~~ Claims 1 ~~to 3~~ wherein the reducing agent is 2-mercaptoethanol or dithiothreitol.

5. (Amended) The nucleic acid isolation kit of ~~any one of~~ Claims 1 ~~to 4~~ wherein the coprecipitant is glycogen or dextran.

6. (Amended) The nucleic acid isolation kit of ~~any one of~~ Claims 1 ~~to 5~~ wherein the protein denaturant is guanidine thiocyanate.

10. (Amended) The method of ~~any one of~~ Claims 7 ~~to 9~~ comprising adding a protein denaturant during alcohol precipitation.

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Serial No. **unknown**

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11. (Amended) The method of ~~any one of Claims 7 to 10~~ wherein the biological component is a body fluid or a blood product.

12. (Amended) The method of ~~any one of Claims 7 to 10~~ wherein the biological sample has a volume of 30 μ l to 100 μ l.

13. (Amended) The method of ~~any one of Claims 7 to 11~~ comprising no step of adding a salt.

14. (Amended) The method of ~~any one of Claims 7 to 12~~ wherein said steps are performed in a single 0.5 ml tube.

progress of HIV decrease in patients undergoing AIDS therapy.

Currently, an indirect detection method based on HIV antigen-antibody reaction has been established and become a major diagnostic means in laboratories. However, detection based on antigen-antibody reaction has been associated with the problem of a window period especially in screening. This is a time-lag between antigen infection and antigen or antibody production. For early clinical diagnosis of HIV infection, it is important to accurately, directly and rapidly detect the presence of HIV RNA in blood as an alternative to the indirect method based on antigen-antibody reaction. It is highly desirable to develop a new detection method that can be performed on smaller amounts of samples with higher sensitivity and at reduced cost and with a shortened window period. It is necessary to diagnose HIV infection from very small amounts of samples especially in infection in children or vertical infection.

Direct and rapid assay of HIV itself has become possible with the new introduction of molecular biological techniques such as polymerase chain reaction (PCR) or screening, for example, into clinical applications (Gerald Schochetman and John J. Sninsky (1991), "Direct Detection of Human Immunodeficiency Virus Infection Using the Polymerase Chain Reaction" Springer-Verlag pp. 90-110; Janet S. Bootman, Pete A. Kitchin (1994), J. Virological Methods, pp. 1-8; Anne-Mieke Vandamme, Sonia Van Dooren, Wessel Kok, Patrick Goubau, Katrien Fransen, Tim Kievits,

Jean-Claude Schmit, Erik De Clercq, Jan Desmyter (1995), J. Virological Methods pp. 121-132; E. Lyamuya, U. Bredberg-Raden, J. Albert, O. Grankvist, V. Msangi, C. Kagoma, F. Mhalu, and G. Biberfeld (1997), J. Clinical Microbiology,

5 pp. 278-280). Such nucleic acid detection systems combining nucleic acid isolation with nucleic acid amplification reactions such as PCR consist of three distinct processes: i.e., nucleic acid isolation, amplification and detection. Nucleic acid isolation is an
10 important process for subsequent amplification and detection, and if a nucleic acid isolation technique can be improved, the efficiency of amplification and detection will increase.

Currently, several types of total RNA isolation
15 methods are known and a plurality of kits are commercially available (John M. Chirgwin, Alan E. Przybyla, Raymond J. MacDonald and William J. Rutter (1979), Biochemistry pp. 5294-5299; Osamu Yamada, Toshiya Matsumoto, Masahiro Nakashima, Shinobu Hagari, Toshio Kamahora, Hiroshi Ueyama,
20 Yuichiro Kishi, Hidetoshi Uemura and Takashi Kurimura (1990), J. Virological Methods pp. 203-210).

However, these methods have disadvantages including the use of organic solvents such as phenol or chloroform to remove proteins and the necessity of changing tubes many
25 times during the isolation process. Further, they are not ideal for isolation from very small amounts of samples, especially blood products or blood samples from children. They also have the disadvantage that costs add up when a

number of samples are assayed in screening or the like.
Thus, it has become increasingly important to develop a
rapid and inexpensive HIV RNA detection system.

In recent years, several types of methods for
5 isolating a nucleic acid, especially RNA have come to be
known such as the guanidinium cyanate-phenol-chloroform
(AGPC) method (Piotr Chomczynski, Nicolette Sacchi (1987),
Analytical Biochemistry pp. 156-159) and methods using
CsCl₂ or a resin (Maniatis T. et al.: Molecular Cloning: A
10 laboratory manual, 2nd ed., Cold Spring Harbor 1989).
Japanese Patent Public Disclosure No. 236499/1995
incorporated herein as reference discloses an improved AGPC
method for isolating a viral nucleic acid, in which the
isolation process can be completed in a single tube without
15 using an organic solvent.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a
nucleic acid isolation kit comprising a reducing agent, a
coprecipitant and a protein denaturant characterized in
20 that it contains no protease.

The nucleic acid isolation kit of the present
invention is preferably an RNA isolation kit.

Another object of the present invention is to provide
a method for isolating a nucleic acid from a biological
25 sample, comprising:

i) incubating the biological sample with a reducing
agent, a coprecipitant and a protein denaturant to degrade
and denature proteins and other contaminants in the

that it contains no protease.

A key feature of the present invention is that no protease is used. In conventional methods, non-specific proteases such as proteinase K, pronase and subtilisin are first used to remove proteins from biological samples. In the present invention, nucleic acids can be efficiently isolated only with a coprecipitant and a protein denaturant without using such a protease.

i) Reducing agents

In the present invention, reducing agents include, but are not limited to, 2-mercaptoethanol, dithiothreitol, etc. These reducing agents are used for protection of sulfhydryl groups in proteins or for reductive cleavage of disulfide bonds.

Reducing agents can be preferably used at a concentration of, but not specifically limited to, about 1%. Reducing agents may be added to nucleic acids as premixes in protein denaturants.

ii) Coprecipitants

In the present invention, coprecipitants are not specifically limited to, and any coprecipitants that can function as carriers for coprecipitating nucleic acids are suitable. Preferred examples of coprecipitants are macromolecular polysaccharides such as glycogen and dextran.

Glycogen is a polymer of D-glucose having a molecular weight of one million to several millions and is known as one of the most potent carriers for nucleic acid

precipitation (Steven Tracy (1981), Biochemistry pp. 251-268), and several types of commercially available glycogen can be used for in vitro applications. In the present invention, glycogen from slipper limpet is preferred from
5 the viewpoints of cost and detection sensitivity.

The concentration of coprecipitants is not specifically limited, but as an example, glycogen from slipper limpet is used at 0.1 mg/ml to 2.0 mg/ml, preferably 0.2 mg/ml to 2.0 mg/ml, most preferably about
10 0.2 mg/ml (Example 2).

iii) Protein denaturants

Suitable protein denaturants are those known to be able to solubilize proteins. They include, but not specifically limited to, guanidine thiocyanate and urea.
15 Guanidine thiocyanate is especially preferred. A preferred embodiment of the present invention uses glycogen as a coprecipitant and guanidine thiocyanate as a protein denaturant, and is called the glycogen-guanidinium (GG) method.

20 Protein denaturants can be used at a final concentration of 3 M to 7 M, preferably 4 M to 6 M, most preferably approximately 4.5 M. Preferably, protein denaturants at the saturation concentration can solubilize proteins and efficiently reduce RNases that may be included
25 in biological samples particularly in the case of isolation of RNA.

Preferably, protein denaturants are added to reactants after biological samples are mixed with

coprecipitants. As described above, they may be added as premixes with reducing agents to reaction solutions.

Further, without ant limitation, protein denaturants can be optionally added to an alcohol during alcohol precipitation at the final stage of the isolation process to more clearly remove contaminant proteins especially in the case of samples containing a lot of contaminants such as blood products. Protein denaturants are added to an alcohol during the alcohol precipitation step preferably at 0.5 M to 2.0 M, most preferably about 0.9 M (Example 3).

In the present invention, there is no special need to externally add a salt during the nucleic acid isolation process, and nucleic acid isolation kits specially require no salt. Moreover, the pH need not be specially externally adjusted.

Nucleic acid isolation methods

The present invention also provides methods for isolating a nucleic acid from a biological sample.

Methods of the present invention comprises:

1) incubating the biological sample with a reducing agent, a coprecipitant and a protein denaturant to degrade and denature proteins and other contaminants in the biological sample without using a protease, and

ii) directly performing alcohol precipitation with a lower alcohol.

In the present invention, nucleic acid isolation can be normally made from biological samples in a volume of about 50 μ l, preferably 30 μ l to 100 μ l. This means that

significantly smaller amounts of biological samples are required than conventional methods.

According to a preferred but non-limitative embodiment, a biological sample is mixed with a coprecipitant and then a reducing agent and a protein denaturant are added in step i).

In step i), incubation takes place at 55°C - 65°C, preferably about 60°C for 5 minutes to 15 minutes, preferably about 10 minutes. Most preferably, incubation takes place at about 60°C for about 10 minutes. Incubation can be performed in an apparatus such as, but not limited to, an incubator, PCR apparatus (eg, Thermal Cycler® from Perkin-Elmer), etc.

In step ii), the reaction mixture obtained in step i) is then combined with a lower alcohol to precipitate a nucleic acid. Alcohol precipitation can be performed by known techniques. Suitable alcohols include isopropanol and ethanol or the like. Preferably, isopropanol is added at a final concentration of 40% or more or ethanol is added at a final concentration of 70% or more. Then, the mixture may be cooled at -70°C to 4°C after addition of a lower alcohol to promote efficacy of salting out.

A protein denaturant may be added during alcohol precipitation as described above. This can remove contaminants precipitating with a target nucleic acid.

Alcohol precipitation is followed by centrifugation at 14,000 x g to 19,000 x g for 5 minutes to 15 minutes. Centrifugation may be performed at room temperature or

under cooling at about 4°C. After centrifugation, the supernatant is decanted or sucked to recover the precipitated nucleic acid.

Finally, the obtained nucleic acid can be washed with 70% ethanol, for example, and redissolved in a suitable solution into a ready-to-use state.

All the steps of the isolation process described above can be performed in a single tube. This can prevent contamination which may otherwise be caused by a change of tubes during the isolation process. This is especially important particularly when the nucleic acid is amplified by PCR or the like. Moreover, the isolation process of the present invention can be performed in a 0.5 ml tube in contrast to conventional methods that required a tube of at least 1.5 ml or more in size. Thus, the necessary amount of each reagent can be reduced and also the nucleic acid redissolved in a 0.5 ml tube can be directly used for amplification reaction.

A preferred but non-limitative embodiment of the present invention is shown in Example 1 and Fig. 2.

Nucleic acid isolation methods of the present invention described above can be applied to isolate both DNA and RNA. Preferably, they can be used for RNA isolation. In addition to RNA of the AIDS virus HIV described in the examples below, they can be clinically applied to type C hepatitis virus (HCV), influenza virus, type A hepatitis virus and the like. In addition to blood samples such as serum and plasma, they are also useful for

soluble liquid samples such as cerebrospinal fluid, saliva, semen and urine. They can also be used for powdered blood products derived from plasma or serum so that they have wide applications. Particularly, they are effective for small amounts of samples such as samples from children or powdered blood products.

Nucleic acids isolated by methods of the present invention can be subsequently amplified and detected, if necessary. That is, methods of the present invention can be combined with amplification techniques such as PCR to detect even very small amounts of nucleic acids at low concentrations that could not be detected by conventional methods.

Amplification

15 (1) Reverse transcription reaction

When the target nucleic acid is an RNA such as viral RNA, the RNA can be reversely transcribed with a reverse transcriptase capable of converting the RNA into cDNA directly in the tube used for isolation. That is, the RNA isolated by methods of the present invention is highly pure and contains no substances inhibiting reverse transcription reaction. Suitable reverse transcriptases include, for example, a reverse transcriptase derived from avian myeloblastosis virus used in Example 1 described below.

25 (2) Amplification of nucleic acids

Nucleic acids isolated by methods of the present invention can be amplified by known nucleic acid amplification reactions such as polymerase chain reaction

provide accurate, direct and rapid diagnosis for virus infection, for example. The GG method of the present invention can be applied not only to the field of research but also to the clinical field.

5 The following examples further illustrate the present invention but are not intended to limit the technical scope of the invention. Those skilled in the art can readily add modifications/changes to the present invention on the basis of the description of the specification, and those
10 modifications/changes are included in the technical scope of the present invention.

EXAMPLES

In the examples of the present invention, the following materials were used unless otherwise specified.

15 Materials

(1) Samples

In both AMPLICOR HIV-1 MONITOR® Test Kit (Roche) and NASBA Amplification System (Organon Teknika, Boxtel, Netherlands), 26 samples containing high copy numbers of HIV-1 RNA (30,000 copies or more / ml) and 47 samples containing low copy numbers below the detection limit (1,000 copies or less / ml) were used.

(2) Glycogen

Oyster glycogen (Sigma), slipper limpet glycogen (Sigma), rabbit glycogen (Sigma) or bovine glycogen (Sigma) was used.

(3) RNA isolation methods used as controls

The following commercially available 7 kits/methods

were used as controls of the present invention in the examples.

TRIZOL LS®

ISOGEN LS® (Nippon Gene)

5 RNA Isolation Technique (Stratagene)

SepaGene-RV® (Sanko Junyaku)

NASBA RNA Isolation Kit (Organon Teknika)

Smitest® (SUMITOMO METAL INDUSTRIES., LTD)

Cartrimox (Iowa Biotechnology).

10 Example 1: RNA isolation by the GG method and amplification and detection of the isolated RNA

HIV-1 RNA was isolated, amplified and detected from HIV-1-containing samples by the following procedures.

a. RNA isolation

15 RNA was isolated from samples following the scheme shown in Fig. 2 as an example of the GG method of the present invention.

In a 0.5 ml tube, 1 µl of glycogen (10 mg/ml) was added to 50 µl of each sample and the tube was agitated.

20 The tube was incubated at 60°C for 10 minutes with 150 µl of 6 M guanidine thiocyanate containing 1% 2-mercaptoethanol (2ME). Then, 200 µl of isopropanol was added. The tube was centrifuged at 15,000 g for 15 minutes at room temperature, and then the supernatant was removed.

25 400 µl of 70% ethanol containing 0.9 M guanidine thiocyanate was added. The tube was centrifuged at 15,000 g for 5 minutes at room temperature, and then the supernatant was removed. 400 µl of 70% ethanol was added.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040 1

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Table 1

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amplification by a conventional PCR method.

i) Initially, 2 drops of mineral oil was added to a 0.5 ml tube containing the above isolated RNA dissolved in 10 μ l of sterile water and the tube was momentary spun.

5 Then, the tube was incubated at 80°C for 10 minutes in a Thermal Cycler® (Perkin-Elmer) to destroy any RNA complexes or secondary structures that may disturb priming in PCR. After 10 minutes, the tube was immediately transferred into ice to stop the reaction and momentary spun.

10 ii) Then, 15 μ l of a mixture for RT having the composition shown in Table 2 below was added and the tube was vortexed and momentary spun.

Table 2. Composition of mixture for RT

15	H ₂ O	7.13
	x 10 PCR buffer	2.5
	10 mM MgCl ₂	2.0
	10 mM DTT	0.5
	2.5 mM dNTP	1.5
20	1 μ l/ml Primer 1 (outer)	0.25
	1 μ l/ml Primer 2 (outer)	0.25
	10 U/ μ l RNase inhibitor	0.77
	2.5 U/ μ l Reverse transferase (AMVRT)	0.1
	Total	15.00 μ l

25

The tube was incubated in a warm bath (42°C) for 60 minutes to synthesize cDNA from mRNA. Then, the tube was momentary spun.

iii) Then, the tube was incubated at 99°C for 6 minutes in a Thermal Cycler to denature double stranded mRNA-cDNA into single strands. After 6 minutes, the tube was immediately transferred into ice to stop the reaction and momentarily spun.

1v) Then, 75 μ l of a mixture for first PCR having the composition shown in Table 3 below was added and the tube was vortexed and momentary spun.

Table 3. Composition of mixture for first PCR

	H ₂ O	58
	x 10 PCR buffer	7.5
	10 mM MgCl ₂	3.0
	2.5 mM dNTP	4.5
15	1 µl/ml Primer 1 (outer)	0.75
	1 µl/ml Primer 2 (outer)	0.75
	Taq polymerase	0.5
	<hr/>	
	Total	75.00 µl

20 First PCR was performed in a Thermal Cycler. Specifically, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute were performed. Then, the tube was incubated at 72°C for 10 minutes and then, the reaction was stopped at 4°C.

25 v) Then, 10 μ l of the first PCR product obtained in
iv) and mineral oil were added to a fresh 0.5 ml tube, and
the tube was momentarily spun. Then, the tube was incubated
at 99°C for 6 minutes in a Thermal Cycler to denature

double-stranded DNA into single strands. After 6 minutes, the tube was immediately transferred into ice to stop the reaction and momentarily spun.

vi) Then, 90 μ l of a mixture for second PCR having the composition shown in Table 4 below was added and the tube was vortexed and momentarily spun.

Table 4. Composition of mixture for second PCR

	H ₂ O	66.5
10	x 10 PCR buffer	10
	10 mM MgCl ₂	5
	2.5 mM dNTP	6
	1 μ l/ml Primer 3 (inner)	1
	1 μ l/ml Primer 4 (inner)	1
15	Taq polymerase	0.5
	Total	90.0 μ l

Second PCR was performed in a Thermal Cycler. Specifically, 30 or 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute were performed. Then, the tube was incubated at 72°C for 10 minutes and then, the reaction was stopped at 4°C.

c. Detection

Electrophoresis and staining

Following amplification, PCR products were electrophoresed. Specifically, a dye was added to the second PCR product and an aliquot of 10 μ l was loaded on a 5% acrylamide gel or a 2% agarose gel and electrophoresed.

primer pair, 40 cycles with the gag primer pair and 30 cycles with the pol primer pair, respectively. Lanes 1-5 in each panel represent bands at glycogen concentrations of 0 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 1 mg/ml and 2 mg/ml, respectively. Lane 6: positive control (P); lane 7: negative control (N); M: molecular weight marker (Roche).

As shown in Fig. 1, bands were detected at glycogen concentrations of 0.2 mg/ml to 2 mg/ml (lanes 3-5) in each case. Therefore, glycogen can function as a carrier at a concentration of about 0.2 mg/ml or more.

Example 3: Optimal concentration of guanidine thiocyanate used for RNA isolation

The concentration of guanidine thiocyanate added to 70% ethanol during the final ethanol washing step was changed to determine the optimal concentration from the viewpoint of isolation efficiency.

Specifically, samples containing low copy numbers of HIV-1 defined above were used to isolate RNA by the GG method of the present invention described above except that the concentration of guanidine thiocyanate added to 70% ethanol during the final ethanol washing step was changed to 0.0 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M and 1.0 M.

As a result, small amounts of contaminants remained as masses in the final RNA at a guanidine thiocyanate concentration of 0.8 M or less but disappeared at 0.9 M or more.

Example 4: Comparison of RNA isolation efficiency

The efficiency of the GG method of the present

invention was compared with those of commercially available known RNA or DNA isolation kits on HIV samples.

Specifically, seven widely used commercial RNA or DNA isolation kits were adopted as controls to perform nucleic acid isolation according to the ordinary protocol of each kit. For each kit, 26 high copy-number samples and 47 low copy-number samples described above were used. Nucleic acids were amplified by PCR with the gag primer pair and/or pol primer pair described above and detected as described above.

The results are shown in Table 5 below.

Table 5

	Isolation	GG method	A	B	C	D
	method					
15	High copy	26/26	10/26	12/26	12/26	10/26
		26/26	10/26	12/26	12/26	10/26
		26/26	11/26	11/26	12/26	9/26
		(100)	(39.7)	(44.9)	(46.2)	(37.2)
20	Low copy	38/47	0/47	0/47	0/47	0/47
		38/47	0/47	0/47	0/47	0/47
		37/47	0/47	0/47	0/47	0/47
		(80.2)	(0)	(0)	(0)	(0)

	Isolation method	E	F	G
5	High copy	10/26	26/26	26/26
		10/26	26/26	26/26
		10/26	26/26	26/26
		(38.5)	(100)	(100)
10	Low copy	0/47	33/47	36/47
		0/47	33/47	36/47
		0/47	32/47	35/47
		(0)	(69.5)	(75.9)

In high copy-number samples, detection was about 35% to about 50% with control kits A to E and 100% with F or G kit. In contrast, the method of the present invention achieved detection of 100%. In low copy-number samples, detection was 0% with control kits A to E and about 70% with F or G kit in contrast to the method of the present invention that achieved detection of 80.2% even in low copy-number samples.

This demonstrates that the present invention shows significantly excellent isolation efficiency as compared with many existing RNA isolation methods.

Example 5: Identification of the sequences of amplified nucleic acids

Amplified samples were sequenced.

Specifically, an automated sequencer ABI-PRISM® 310 (Perkin-Elmer) was used for direct sequencing as

recommended by the manufacturer. The target region in this Example is the gag or pol region of HIV-1. The reference sequence was retrieved from the annual report of the Los Alamos National Laboratory. Phylogenetic trees were used
5 for data analysis.

The results on PCR products using the gag primer pair are shown in Fig. 3 and SEQ ID NOS: 10-19. The nucleotide sequences of nucleic acids can be determined in all the samples subjected to RNA isolation by the GG method of the
10 present invention. Sequencing of 10 samples revealed a common sequence of subtype E (Fig. 3). Sequence data show that all the 10 samples tested have a 90% or more homology with the common sequence (SEQ ID NO: 9) in the gag region of HIV-1 (291 bp). In Fig. 3, bases identical to those of
15 the common sequence are shown by "-".

This verifies that each sample subjected to nucleic acid isolation contains HIV-1 RNA and that the presence of HIV-1 was detected by isolation and amplification of the RNA.

20 EFFECT OF THE INVENTION

We compared known isolation methods using commercial kits with a method of the present invention. The results showed that the method of the present invention was the most excellent isolation method from the viewpoints of
25 detection sensitivity, economy and time reduction.

Conventional methods using CsCl₂ or a resin were suitable in terms of time reduction, but inferior to the method of the present invention in detection sensitivity.

The method of the present invention is safer than the AGPC method because it uses neither phenol nor chloroform. Moreover, the necessary period is within 60 minutes, which is shorter than required by conventional methods because
5 any organic solvents such as phenol and chloroform are not used. The method of the present invention can be performed more inexpensively on small amounts (about 50 μ l) of samples. All the steps of the method of the present invention can be performed in a 0.5 ml tube with no need to
10 change the tube. Thus, processes from nucleic acid isolation to amplification can be completed in a single step, whereby the risk of contamination of reaction products is decreased.

Without being bound to any theory, the excellent
15 isolation efficiency of the method of the present invention is partially attributed to the use of glycogen as a carrier of nucleic acids. The present invention is particularly useful for screening small amounts of samples such as blood products or blood samples from children, for example, and
20 for following up patients under AIDS therapy, and it is useful for not only the field of research but also clinical applications.

CLAIMS

1. A nucleic acid isolation kit comprising a reducing agent, a coprecipitant and a protein denaturant characterized in that it contains no protease.
2. The nucleic acid isolation kit of Claim 1, which is salt-free.
3. The nucleic acid isolation kit of Claim 1 or 2 wherein the nucleic acid is RNA.
4. The nucleic acid isolation kit of any one of Claims 1 to 3 wherein the reducing agent is 2-mercaptoethanol or dithiothreitol.
5. The nucleic acid isolation kit of any one of Claims 1 to 4 wherein the coprecipitant is glycogen or dextran.
6. The nucleic acid isolation kit of any one of Claims 1 to 5 wherein the protein denaturant is guanidine thiocyanate.
7. A method for isolating a nucleic acid from a biological sample, comprising:
 - i) incubating the biological sample with a reducing agent, a coprecipitant and a protein denaturant to degrade and denature proteins and other contaminants in the biological sample without using a protease, and
 - ii) directly performing alcohol precipitation with a lower alcohol.
8. The method of Claim 7 wherein incubation takes place at 55°C to 65°C for 5 minutes to 15 minutes in step i).
9. The method of Claim 8 wherein incubation takes place at about 60°C for about 10 minutes in step i).

10. The method of any one of Claims 7 to 9 comprising adding a protein denaturant during alcohol precipitation.
11. The method of any one of Claims 7 to 10 wherein the biological component is a body fluid or a blood product.
12. The method of any one of Claims 7 to 10 wherein the biological sample has a volume of 30 μ l to 100 μ l.
13. The method of any one of Claims 7 to 11 comprising no step of adding a salt.
14. The method of any one of Claims 7 to 12 wherein said steps are performed in a single 0.5 ml tube.

ABSTRACT

The present invention provides a nucleic acid
isolation kit comprising a reducing agent, a coprecipitant
and a protein denaturant characterized in that it contains
5 no protease, and a method for isolating a nucleic acid
using said kit.

Fig. 1

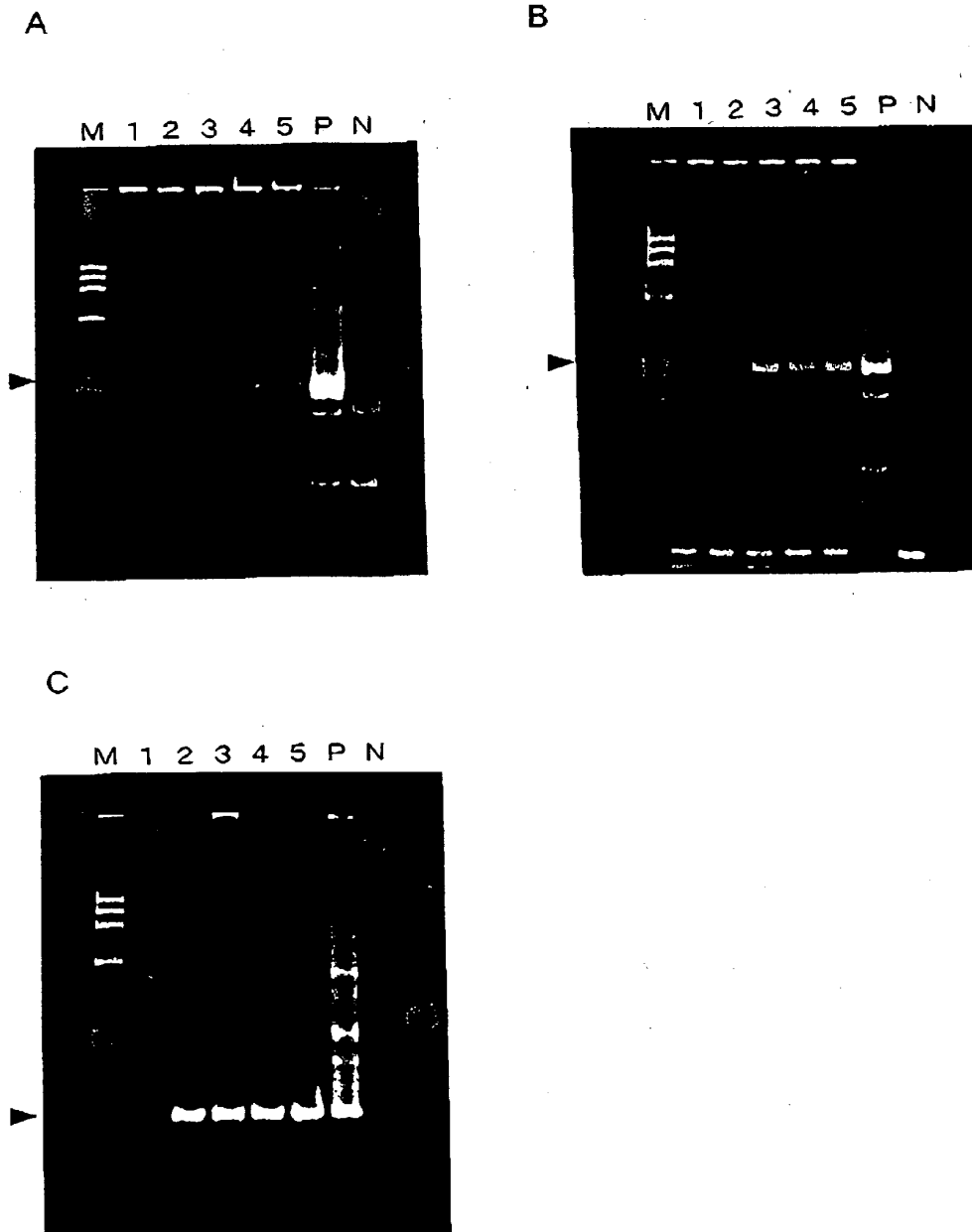


Fig.3

COMMON SEQUENCE E	10	20	30	40	50	60	70
SAMPLE 1	CTATGCAaAT	gtTAAAGAT	aCCATCAATG	agGhaGCTGC	AGaATgGGAC	agGgtACATC	CAGTACATGC
SAMPLE 2	-C-----	-A-----	-A-----	-A-----	-T-----	-C-----	-C-----
SAMPLE 3	-C-----	-A-----	-A-----	-A-----	-T-----	-C-----	-C-----
SAMPLE 4	-C-----	-A-----	-A-----	-A-----	-T-----	-C-----	-C-----
SAMPLE 5	-C-----	-A-----	-A-----	-A-----	-T-----	-C-----	-A-----
SAMPLE 6	-C-----	-A-----	-A-----	-A-----	-T-----	-C-----	-C-----
SAMPLE 7	-C-----	-G-----	-G-----	-G-----	-T-----	-C-----	-C-----
SAMPLE 8	-C-----	-A-----	-A-----	-A-----	-T-----	-C-----	-C-----
SAMPLE 9	-C-----	-A-----	-A-----	-A-----	-T-----	-C-----	-C-----
SAMPLE 10	-C-----	-C-----	-C-----	-C-----	-T-----	-C-----	-C-----
COMMON SEQUENCE E	80	90	100	110	120	130	140
SAMPLE 1	AGGGCCTatt	ccACCaGGGc	AgATGAGaGA	ACCAAggGGA	agTGACATAG	CAGGaactAC	TAGTACcCTT
SAMPLE 2	-A-----	-G-----	-G-----	-G-----	-A-----	-A-----	-A-----
SAMPLE 3	-A-----	-G-----	-G-----	-G-----	-A-----	-G-----	-A-----
SAMPLE 4	-A-----	-G-----	-G-----	-G-----	-A-----	-G-----	-A-----
SAMPLE 5	-A-----	-G-----	-G-----	-G-----	-A-----	-G-----	-A-----
SAMPLE 6	-A-----	-G-----	-G-----	-G-----	-A-----	-G-----	-A-----
SAMPLE 7	-A-----	-G-----	-G-----	-G-----	-A-----	-G-----	-A-----
SAMPLE 8	-A-----	-G-----	-G-----	-G-----	-A-----	-G-----	-A-----
SAMPLE 9	-A-----	-G-----	-G-----	-G-----	-A-----	-G-----	-A-----
SAMPLE 10	-A-----	-G-----	-G-----	-G-----	-A-----	-G-----	-A-----
COMMON SEQUENCE E	150	160	170	180	190	200	210
SAMPLE 1	caAGRACaa	TaggaTgGAT	GACaagCAAT	CCACCTatcc	CaGTgGGaGA	cATcTATaAa	
SAMPLE 2	-G-----	-A-----	-A-----	-A-----	-A-----	-A-----	
SAMPLE 3	-G-----	-A-----	-A-----	-A-----	-A-----	-A-----	
SAMPLE 4	-G-----	-A-----	-A-----	-A-----	-A-----	-A-----	
SAMPLE 5	-G-----	-A-----	-A-----	-A-----	-A-----	-A-----	
SAMPLE 6	-G-----	-A-----	-A-----	-A-----	-A-----	-A-----	
SAMPLE 7	-G-----	-A-----	-A-----	-A-----	-A-----	-A-----	
SAMPLE 8	-G-----	-A-----	-A-----	-A-----	-A-----	-A-----	
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Case No. _____

Nixon & Vanderhye P.C. (12/87)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

KITS FOR EXTRACTING NUCLEIC ACID AND METHOD OF EXTRACTING NUCLEIC ACID BY USING THE KITS

the specification of which (check applicable box(es)):

- ☐ is attached hereto
☐ was filed on _____

as U.S. Application Serial No. _____

☒ was filed as PCT International application No. PCT/JP00/05170 on August 2, 2000

and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number
190633/1999Country
JapanDay/Month/Year Filed
5/7/1999

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number

Date/Month/Year Filed

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):
Application Serial No.

Day/Month/Year Filed

Status: patented
pending, abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed), and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32108; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffrey H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33483; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834; B. J. Sedoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334.

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FOR ADDITIONAL INVENTORS, check box ☒ and attach sheet with same information and signature and date for each.

268050

Case No. _____

Nixon & Vanderhye P.C. (12/97)

RULE 63 (37 C.F.R. 1.63)
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FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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	Post Office Address: (Zip Code)			
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268050

SEQUENCE LISTING

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NAITOU Toshikuni

National Institute of Infectious Diseases

TAKEDA Yoshifumi

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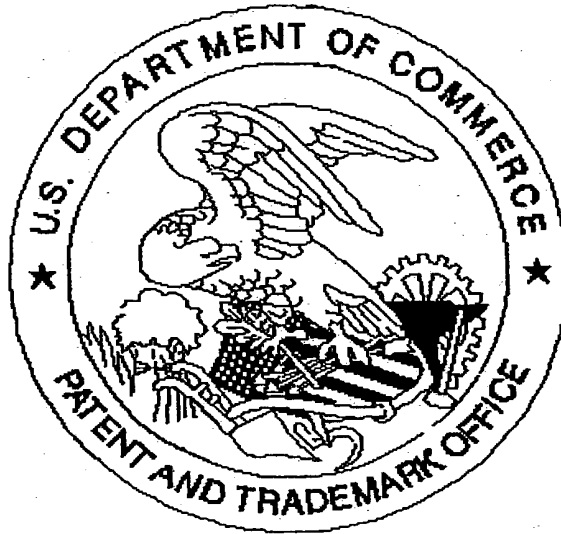
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